**FBS09- Differential Organic DNA Extraction**

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# 1. Scope

1.1. This procedure describes the isolation of deoxyribonucleic acid (DNA) from biological specimens presumed to have sperm, recovered from evidentiary items for nuclear DNA typing.

# 2. Background

2.1. This procedure, in which epithelial and sperm cells are separated, is a modified version of the Organic DNA Extraction (FBS08) method. The Differential Extraction procedure preferentially lyses the non-sperm cells (EF) leaving the sperm cells (SF) intact. The remaining sperm cells are treated with dithiothreitol (DTT) which breaks the protein disulfide bonds that make up the cell membranes. This method will be used on any sample where the presence of spermatozoa is suspected or has been confirmed.

# 3. Safety

3.1. Wear personal protective equipment (e.g., lab coat, gloves, mask, eye protection), when carrying out standard operating procedures (SOPs).

3.2. Read Safety Data Sheets (SDSs) to determine the safety hazards for chemicals and reagents used in the SOPs.

# 4. Materials Required

4.1. Digest Buffer (FBR35)

4.2. Proteinase K (10 mg/ml) (FBR36)

4.3. Phenol/Chloroform/Isoamyl Alcohol (PCI)

**NOTE**: This reagent and its waste must be handled in a fume hood. Allow to equilibriate to room temperature before use.

4.4. 1.0 M DTT (FBR38)

4.5. TE (Tris EDTA) Buffer or autoclaved deionized water (diH2O) (FBR06)

**NOTE**: Never use solutions directly from the stock bottles. Use Reagent SOPs for preparation and labeling instructions.

4.6. Microcentrifuge

4.7. 1.5 mL and 2.0 mL Microcentrifuge tubes

4.8. Microcon devices

# 5. Standards and Controls

5.1. At least one reagent blank (RB) (e.g., extraction control) must be prepared and processed in parallel with each set of evidentiary specimens processed for DNA typing purposes. The RB(s) is comprised of all the reagents used in the analytical process and is carried through the same extraction, quantitation, amplification and detection procedure(s) as the evidence samples. If more than one extraction method is used, then at least one RB must be processed for each type of procedure.

5.2. For differentials, the RB created in conjunction with the isolation of the female fraction is designated as the non-sperm fraction (RB#EF or BK#.E in the Sample Tracking and Control Solutions (STACS)). The RB created in conjunction with the isolation of the male fraction is designated as the sperm fraction (RB#SF or BK#.S in the STACS). The RB will always be the last sample processed in a set.

5.3. If a recombined sample (pellet and substrate) from p30 or sperm search testing is to be extracted, its associated Negative Control(s) will be processed in addition to the extraction RB. The Negative Control(s) will be created in STACS as an RB and include a unique identifier in the Lot Number location to differentiate it from the extraction RB. This Negative Control will be comprised of all the reagents used to prepare the sample for serology testing in addition to all the reagents

used in the analytical process. It will be carried through the same lab processes, as applicable, as the evidence sample with which it was associated.

5.4. Samples suspected of containing low levels of DNA should be extracted separately from samples suspected of containing high levels of DNA whenever practicable.

5.5. In order to maintain a separation in time and space between questioned and known samples:

5.5.1. At no time will questioned and known samples be simultaneously incubating in the same heat block.

5.5.2. At no time will questioned and known samples be simultaneously extracted in the organic fume hood.

# 6. Procedures

6.1. Place each sample into a labeled, sterile 2.0 mL microcentrifuge tube.

6.2. **The order and labeling of initial tubes and final elution tubes must be witnessed by a second trained individual.** The witness step will be captured in the Batch Comments of the appropriate STACS documentation.

6.3. To each sample tube, pipette 400 µL Digest Buffer and 12 µL of Proteinase K (10 mg/mL) solution. Vortex and quick-spin in a microcentrifuge.

6.4. **NOTE**: The Digest Buffer and Proteinase K may be aliquoted as a master mix. If the master mix method is used, this aliquot expires at the end of the prepared date.

6.5. Incubate the samples on the heat block either at 37ºC for 2 hours to overnight or at 56ºC for 1 hour.

6.6. After incubation, vortex and spin-down the sample tubes. Transfer the substrate to a filterless basket in a 2.0 mL microcentrifuge tube and spin for 3 – 5 minutes at maximum speed. Discard the substrate and basket.

6.7. Remove the supernatant from the samples, being careful not to disturb the sperm pellet (approximately 50 µL remaining in the tube). Place the supernatant (non-sperm fraction [EF]) into a new appropriately labeled sterile microcentrifuge tube. Set non-sperm fraction [EF] extracts to the side and proceed to next step.

**NOTE**: EF tubes can be processed immediately (see step 6.14) or can be stored at 4°C (for a maximum of overnight) for simultaneous processing with sperm fractions (SF).

6.8. Wash the sperm pellet by re-suspending in 500-1000 µL of TE Buffer or di$H_2O$. Vortex and spin the samples in a microcentrifuge for 3-5 minutes at maximum velocity. Remove and discard the supernatant, being careful not to disturb the sperm pellet (up to 50 µL may be left in the tube).

6.9. Repeat step 6.8 an additional 2 times for a total of 3 washes of the sperm pellet.

6.9.1. **NOTE**: The wash step can be repeated an additional 1 to 5 times depending upon the nature of the sample.

6.10. After the final spin, remove and discard all but approximately 50 µL of the supernatant.

6.11. **OPTIONAL**: Resuspend the sperm pellet within the remaining 50 µL of supernatant by gently mixing the sample with a pipette. Remove approximately 4 µL of the sample and spot it on a glass microscope slide. Heat fix cells to the microscope slide following step 6.7 of FBS07 – Microscopic Examination of Spermatozoa by Christmas Tree Stain (Document Control Number: 1577).

6.11.1. **OPTIONAL:** Perform a Christmas Tree Stain using the technique described in steps 6.8 through 6.15 of FBS07 - Microscopic Examination of Spermatozoa by Christmas Tree Stain (Document Control Number: 1577). Proceed to step 6.12 if no epithelial cells are observed. However, if any intact epithelial cells remain, re-digest the sperm pellet by following these additional steps:

6.11.1.1. Add 400 µL of Digest Buffer to re-suspend the sperm pellet.

6.11.1.2. Add 12 µL of Proteinase K (10 mg/mL). Mix gently.

6.11.1.3. Incubate at 37°C for 1 hour.

6.11.1.4. Spin at a maximum velocity for 3-5 minutes in a microcentrifuge. Remove and discard all but approximately 50 µL of the supernatant.

6.11.1.5. Re-suspend the pellet in 500 µL of Digest Buffer and vortex. Spin the sample 3-5 minutes in the microcentrifuge at maximum velocity. Remove and discard all but

approximately 50 µL of the supernatant. Proceed to Step 6.12.

6.12. To the sperm pellet add 400 µL Digest Buffer, 12 µL 1M DTT, and 15 µL Proteinase K (10 mg/mL). The Digest Buffer and Proteinase K may be added to the sample and incubated at 37°C or 56ºC while performing the Christmas Tree Stain. Once a slide is confirmed as containing no epithelial cells, the DTT may be added and the incubation continued.

6.12.1. **NOTE**: The Digest Buffer, Proteinase K and DTT may be aliquoted as a master mix. If the master mix method is used, this aliquot expires at the end of the day.

6.13. Mix the samples gently and incubate on the heat block at 56ºC for 2 hours to overnight. Vortex and spin down.

6.14. In a fume hood add 500 µL of Phenol/Chloroform/Isoamyl Alcohol (PCI) solution to the sperm and non-sperm fractions. Thoroughly mix (vortex or invert by hand) to form a transiently homogeneous suspension.

6.14.1. All nuclear DNA isolation steps in which Phenol/Chloroform/Isoamyl Alcohol reagent is used must be performed in a fume hood.

6.14.2. To ensure a homogeneous solution, the Phenol/Chloroform/Isoamyl Alcohol reagent will be removed from the refrigerator and allowed to equilibrate to room temperature before beginning extractions.

6.15. Microcentrifuge the samples at room temperature for 5 minutes at maximum speed to separate the two phases. Proceed to Step 6.16 if the upper aqueous phase is clear.

6.15.1. **NOTE:** If the aqueous phase is not clear (e.g., cloudy, dark in color or colored from dyes) due to incomplete phase separation, then transfer the upper aqueous phase to a new sterile 1.5 mL microcentrifuge tube. Repeat step 6.14 – 6.15 an additional 2 to 3 times, until the interface is clean and the aqueous phase is clear. (For additional extractions, the lower Phenol/Chloroform/Isoamyl Alcohol layer may be removed and discarded, eliminating the need for a new microcentrifuge tube).

6.16. Assemble the Microcon and label the specimen reservoir.

6.17. Add 100 µL of TE Buffer to the upper reservoir to pre-wet the membrane.

6.18. **NOTE:** When adding sample and/or buffer to specimen reservoir be cautious to avoid touching the filter with the pipette tip.

6.19. Transfer the entire aqueous phase of the samples to the upper reservoir containing the TE Buffer. Microcentrifuge at 500 x g for at least 10 minutes.

6.19.1. Caution will be taken to prevent the filters from exceeding their specified limitations. Excessive g-force may result in leakage or damage to the centrifugal device.

6.20. Remove the upper specimen reservoir from the tube, discard the effluent in the lower reservoir, and re-insert the upper reservoir into the Microcon tube. Alternatively, the upper reservoir may be placed into a new appropriately labeled Microcon tube and the original tube containing the effluent discarded.

6.21. Add 200 µL of TE Buffer to the upper reservoir.

6.22. Microcentrifuge the samples at 500 x g for at least 10 minutes. If any liquid remains, additional spin(s) may be performed.

6.23. Label a new set of Microcon recovery tubes.

6.24. When the fluid has been drawn through the filter, add approximately 10-25 µL of TE buffer to reservoir, and invert the upper reservoirs into the recovery tubes. Microcentrifuge for 3 minutes at 500 x g. Be certain that the caps on the tubes are all facing inward in the microcentrifuge to avoid possible snapping of the caps.

6.24.1. Extended centrifugation (2-3 time longer than guidelines) can lead to dryness. Caution will be taken to prevent the filters from spinning to dryness. If this should occur, vortex for 10-30 seconds after addition of the 10-25 µL of TE buffer, then proceed with recovery.

6.25. Remove and discard filter and close final Microcon tube. The contents of the final Microcon tube will be transferred to an appropriately labeled sterile microcentrifuge tube. Add additional TE buffer, if necessary, to bring the final sample volume to at least 32 µL. **NOTE:** A higher recovery volume of ~ 50 µL is recommended for samples of expected high DNA concentration, e.g. reference samples.

6.26. Reagent Blanks and, if applicable, Serology Negative Controls must be less than or equal to the lowest volume (highest concentration) of an associated sample within an extraction set.

6.27. Store the samples frozen.

6.28. If needed, a sample may be re-concentrated following steps below:

6.28.1. Assemble the Microcon and label the specimen reservoir.

6.28.2. Add 100 µL TE Buffer to upper reservoir to pre-wet membrane.

6.28.3. Add the DNA sample to the upper reservoir, being cautious to avoid touching the filter with the pipette tip.

6.28.4. Repeat steps 6.22 – 6.25.

6.28.5. **NOTE:** If a sample is to be re-concentrated, the associated reagent blank must also be re-concentrated as well.

# 7. Sampling

7.1. Not applicable

# 8. Calculations

8.1. Not applicable

# 9. Uncertainty of Measurement

9.1. Not applicable

# 10. Limitations

10.1. The quantity and quality of the DNA present within any biological material ultimately determines if a nuclear DNA isolation is successful.

10.2. The separation of non-sperm and sperm cell DNA into their respective fractions is not always complete. It is not unusual for sperm cell DNA to be observed in the non-sperm (or epithelial) cell fraction and vice versa. The number of intact cells recovered in a sample and their capacity to endure the abrasive conditions of the differential extraction method is dependent upon the quality of the biological material being tested and the environmental conditions to which it has been subjected. The detection of residual DNA within a given fraction does not prohibit the use of the DNA typing results from that fraction.

10.3. The presence of nuclear DNA in the sperm fraction of a differential extraction is not always a dependable method of determining whether semen or spermatozoa in a particular biological specimen is present. Many factors may influence this determination such as differing profiles in the sperm fraction and non-sperm fraction of the sample and the associated semen screening results (AP (FBS05), p30 (FBS06), microscopic sperm identification (FBS07)).

# 11. Documentation

11.1. Applicable STACS documentation

# 12. References

12.1. Microcon® Centrifugal Filter Devices User Guide. Merck Millipore Ltd., 2018.

12.2. Forensic Biology Unit Quality Assurance Manual

12.3. Sterile Deionized Water (FBR06)

12.4. Digest Buffer (FBR35)

12.5. 10 mg/ml Proteinase K in TE buffer (FBR36)

12.6. 1.0 M Dithiothreitol (DTT) (FBR38)

12.7. Acid Phosphatase Presumptive Chemical Test for the Presence of Seminal Fluid (FBS05)

12.8. P30 Antigen Test for the Presence of Seminal Fluid (FBS06)

12.9. Microscopic Examination of Spermatozoa by Christmas Tree Stain (FBS07)

12.10. Organic DNA Extraction (FBS08)